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ADULT HOUSE FLY FEEDING DETERRENT
FROM NEEM SEEDS

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W. R. Lusby, and H. Finegold^{1/}

ABSTRACT

A compound from an ethanolic extract of neem seeds, Azadirachta indica A. Juss., possessed more feeding deterrence for adult house flies, Musca domestica L., at 0.1 percent on sucrose than did the insect repellent N,N-diethyl-m-toluamide (deet). It also was as active as formulations of two fly repellents, dipropyl 2,5-pyridinedicarboxylate and 3-acetyl-2-(2,6-dimethyl-5-heptenyl)oxazolidine. The compound was identified as salannin, a triterpenoid, previously isolated as a constituent of neem. High-performance liquid chromatography on a reversed-phase column and other reported techniques were used for the isolation.

KEYWORDS: Azadirachta indica, neem, salannin, feeding deterrent, Musca domestica, house fly.

INTRODUCTION

Two reported insect feeding deterrents (antifeedants) have been isolated from the neem tree, Azadirachta indica A. Juss.^{2/} They are the triterpenoids meliantriol (7) and azadirachtin (1, 2, 3, 8, 10). Many other constituents in neem have been identified, but none have been shown to be feeding deterrents.

During a screening of potential feeding deterrents for the adult house fly, Musca domestica L., we found that a 95 percent ethanolic extract of neem seeds caused feeding deterrence to sucrose. The component responsible for

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^{2/} Also referred to as Melia azadirachta L., M. indica Brandis, Margosa tree, or Indian lilac.

this activity was isolated and identified. The degree of adult house fly feeding deterrence of this component was compared with that of two fly repellents, dipropyl 2,5-pyridinedicarboxylate and 3-acetyl-2-(2,6-dimethyl-5-heptenyl)oxazolidine, and with N,N-diethyl-m-toluamide (deet), a repellent for biting flies, chiggers (mites), ticks, mosquitoes, fleas, gnats, and other biting insects. The feeding deterrence of this neem component against adult house flies was also compared with that of a number of natural products and synthetic compounds.

MATERIALS AND METHODS

Solvents

All solvents used for extraction and open-column fractionation were reagent-grade. Methanol for high-performance liquid chromatography (hplc) was hplc-grade.

Chromatography of Plant Material

Fresh neem seeds (5.76 kg) from India were ground and extracted with 95 percent ethanol. The extract was partitioned between methanol and hexane, and the methanol-soluble portion was subjected to chromatography on Florex RVM (Floridin Co., 60-100 mesh) according to Warthen et al. (8).

Thirty fractions were collected and tested for adult house fly feeding deterrence. Of these, the sixth, seventh, and eighth fractions (containing 43.2 g, 35.4 g, and 30.2 g of neem material, respectively) were the most active. The seventh fraction (1.0 g) in a minimum amount of toluene was placed on a 60-g, 50- by 2.4-cm o.d. Florisil (Floridin Co., 60-100 mesh) column, and six 200-ml fractions were then eluted successively with ether and with 1, 3, 5, 25, and 50 percent acetone-ether. The active fractions 2-5 (422 mg) in a minimum of 50 percent toluene-ether were then chromatographed on a 30-g, 30- by 3.2-cm o.d. Bio-Sil HA (Bio-Rad Laboratories, minus 325 mesh) column, and four 100-ml fractions were eluted successively with 50 percent toluene-ether, ether, and 3 and 25 percent acetone-ether.

High-Performance Liquid Chromatography

The active second and third fractions (273.5 mg) were subjected to hplc on a reversed-phase 30- by 0.78-cm i.d. μ Bondapak C-18 column with 65 percent methanol-water at 4 ml/min. Thirty-four 1-ml fractions were collected. A Waters Associates ALC-100 liquid chromatograph equipped with an M-6000 pump, a U6K injector, an M-660 solvent programmer, a Schoeffel Instrument Corp. SF 770 multi-wavelength UV detector, and a Buchler Fractomette 200 fraction collector was used for all hplc. Monitoring at 254 nm was done for all hplc runs. Fractions 21-24 (97 mg) were effective in inhibiting feeding in the adult house fly.

Bioassay

House flies were allowed to emerge from approximately 1,500 pupae in a 25- by 25- by 53-cm sleeve cage, held at 22-27° with fluorescent lighting

from 0800 to 1600 hour. Before the test, flies were provided with water and a mixture of powdered milk and sugar. These were removed approximately 1 hour before the test. Tests were conducted with 3- to 10-day-old flies.

The test materials in acetone were added to sucrose. An even distribution of the test materials on sucrose was obtained by removing the solvent via rotary evaporator. Initially, a concentration of 0.5 percent on sucrose was used; however, materials that proved to be active were further tested at 0.25 and 0.1 percent. Those showing little activity were tested at 1.0 percent.

The treated and untreated sucrose samples were placed on 7.5-cm watch glasses in petri dishes. Counts of flies feeding on the sucrose were made 3 minutes after the petri dishes were placed in the cage. Then the flies were shooed away, and a second count was made 3 minutes later. These two counts were then averaged for a test/control count.

Physical Measurements

The melting point (mp) of the house fly feeding deterrent was taken on a Thomas Hoover Capillary Melting Point Apparatus. The infrared (ir) spectrum (KBr pellet) was recorded on a Perkin-Elmer Model 137 Spectrophotometer. Nuclear magnetic resonance (nmr) spectra in $(\text{CD}_3)_2\text{CO}$ were recorded on a JEOL FX60-Q pulsed Fourier-transform Spectrometer with a 5-mm dual probe with SiMe_4 as the reference. ^1H nmr data were recorded after a single scan at 500-Hz spectral width. ^{13}C nmr data (^1H decoupled) were recorded after 1,400 scans at 4000-Hz spectral width. Electron impact mass spectra (ei-ms) were obtained by gas-liquid chromatography and direct probe with an LKB 9000 GC/MS (LKB Producter AB, Stockholm, Sweden) (120 μA , 70 eV) equipped with a 305- by 0.4-cm i.d. column packed with 0.75 percent SE 30 on silanized acid-washed 100/140 mesh Gas Chrom P. A Varian SS-100 data system was employed to process the mass spectral data. Ei-ms were also obtained on a Hewlett-Packard 5992A GC/MS equipped with a 91.4- by 0.2-cm i.d. column packed with 2 percent OV 101 on 100/120 mesh Chromosorb WHP. The chemical ionization (ci) spectra were obtained by direct probe with a Dupont 491-B GC/MS equipped with a Dupont 21-094 data system. The source temperature was 125°. Isobutane and ammonia were used as ci reagent gases.

Derivatization

The adult house fly feeding deterrent was hydrolyzed, methylated with 14 percent BF_3 in methanol, and acetylated (4). The resulting derivative was then purified via hplc on the reversed-phase 30- by 0.78-cm i.d. μ Bondapak C-18 column with 65 percent methanol-water at 4 ml/min. The purified derivative was subjected to ei-ms on the Hewlett Packard 5992A GC/MS system.

RESULTS AND DISCUSSION

The adult house fly feeding deterrent, mp 167-169° uncorrected (167-170°, lit.) (6), that was isolated via Florex, Florisil, silicic acid, and μ Bondapak C-18 chromatography gave a single spot on silica gel thin layer

chromatography (Pierce-Quantin MQ65, 200 μ m, 1 by 3 inches) with 50 percent chloroform-ethyl acetate as developer.

The spectrum from the Hewlett-Packard 5992A GC/MS (fig. 1) and the spectra obtained from the LKB 9000 indicated a parent ion of mass to charge (m/e) 596 (4) that corresponded to the molecular weight of the neem constituent "salannin" (I, fig. 2) $C_{34}H_{44}O_9$ (5,6). To confirm that this was the parent ion at m/e 596, we recorded ci-ms of this compound with isobutane and ammonia as the reaction gases (fig. 3). An $(M + H)^+$ peak at 597 was obtained with isobutane and an $(M + NH_4)^+$ peak at 614 was obtained with NH_3 .

The ir spectrum (fig. 4) showed ν_{max}^{KBr} 1710 (tiglate ester), 1743 (acetate and methyl esters), and 1653 (olefinic linkage) cm^{-1} (6). The 1H nmr data (fig. 5) showed a spectrum almost identical to that of photosalannin (9) except for the lack of a singlet at 5.99 p/m and of a broad signal at 6.16 p/m that could be assigned to the carbinyl proton and the α proton of the γ -hydroxybutenolide system. Instead, a β -proton (diffuse singlet) at 6.39 p/m and two α -protons (multiplet) at 7.43 p/m of a β -substituted furan ring (6) were present. The ^{13}C nmr data showed a spectrum identical to that obtained by Zanno (9) for salannin.

Hydrolysis, methylation, and acetylation of salannin gave (fig. 2) salannic acid (II), methyl salanninate (III), and methyl salanninate diacetate (IV), respectively (4). The ei-gc/ms of (IV) showed the same peak at m/e 556, parent ion ($C_{31}H_{40}O_9$), that was shown by de Silva et al. (4).

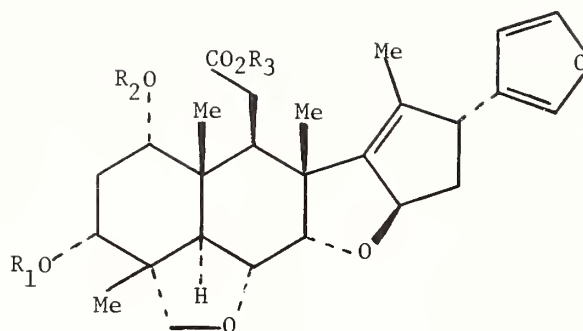
Therefore the adult house fly feeding deterrent is salannin (I).

Table 1 shows the house fly feeding deterrence of a number of natural products, synthetic compounds and mixtures, and salannin. The adult house fly deterrent in neem, identified as salannin (I), compared favorably with the insect repellent N,N-diethyl-m-toluamide (deet) as a feeding deterrent for adult house flies. At 0.1 percent the feeding deterrence of salannin was superior to that of deet. Although deet is a general repellent for biting insects, including flies, this is still a favorable comparison due to the wide use of this repellent. Only the fly repellents, Z8econ 0759 and 0760, which contain 15 percent dipropyl 2,5-pyridinedicarboxylate and 3-acetyl-2-(2,6-dimethyl-5-heptenyl)oxazolidine, respectively, were as active as salannin. However, if these fly repellents in the Z8econ formulations were used undiluted, they might be more active than salannin.

A few of the terpenes, such as cedrene and limonene, showed feeding deterrence but at the higher concentration of 1.0 percent. Limonene, β -caryophyllene, and amorphene showed some feeding deterrence at 0.5 and 1.0 percent. The three essential oils and the terpenes of sweet orange also showed a low level of feeding deterrence at 0.5 and 1.0 percent; this finding may warrant further investigation since these materials are mixtures.



Figure 1.--Gc/ms spectrum of house fly feeding deterrent.



- Figure 2.--I $R_1 = \text{COCH}_3$; $R_2 = \text{COC}_4\text{H}_7$; $R_3 = \text{CH}_3$
 II $R_1 = R_2 = R_3 = \text{H}$
 III $R_1 = R_2 = \text{H}$; $R_3 = \text{CH}_3$
 IV $R_1 = R_2 = \text{COCH}_3$; $R_3 = \text{CH}_3$

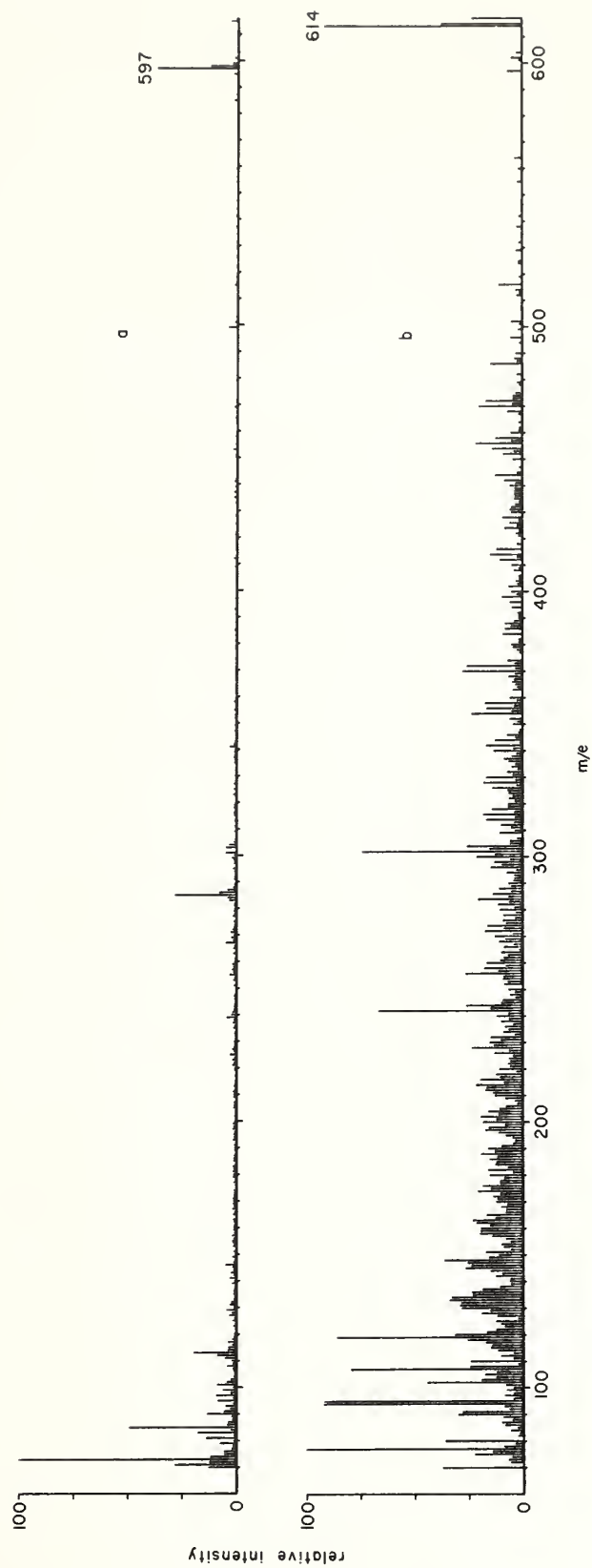


Figure 3.--Ci-ms spectra of house fly feeding deterrent with
(a) isobutane and (b) ammonia as the reaction gases.

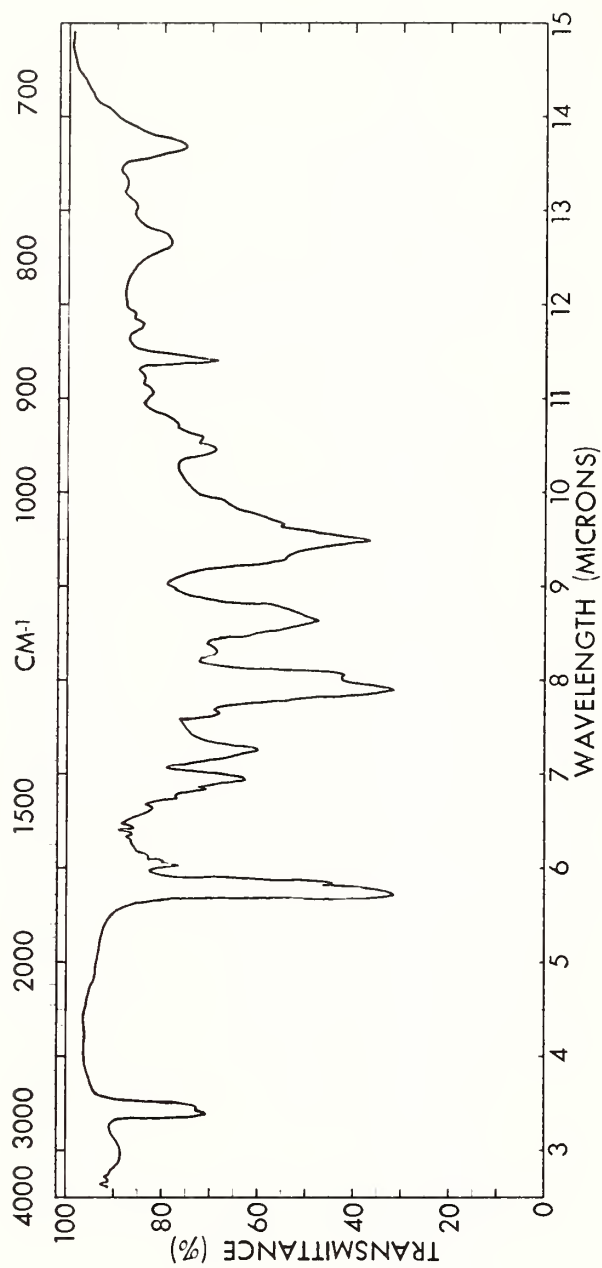


Figure 4.--Ir (KBr) spectrum of house fly feeding deterrent.

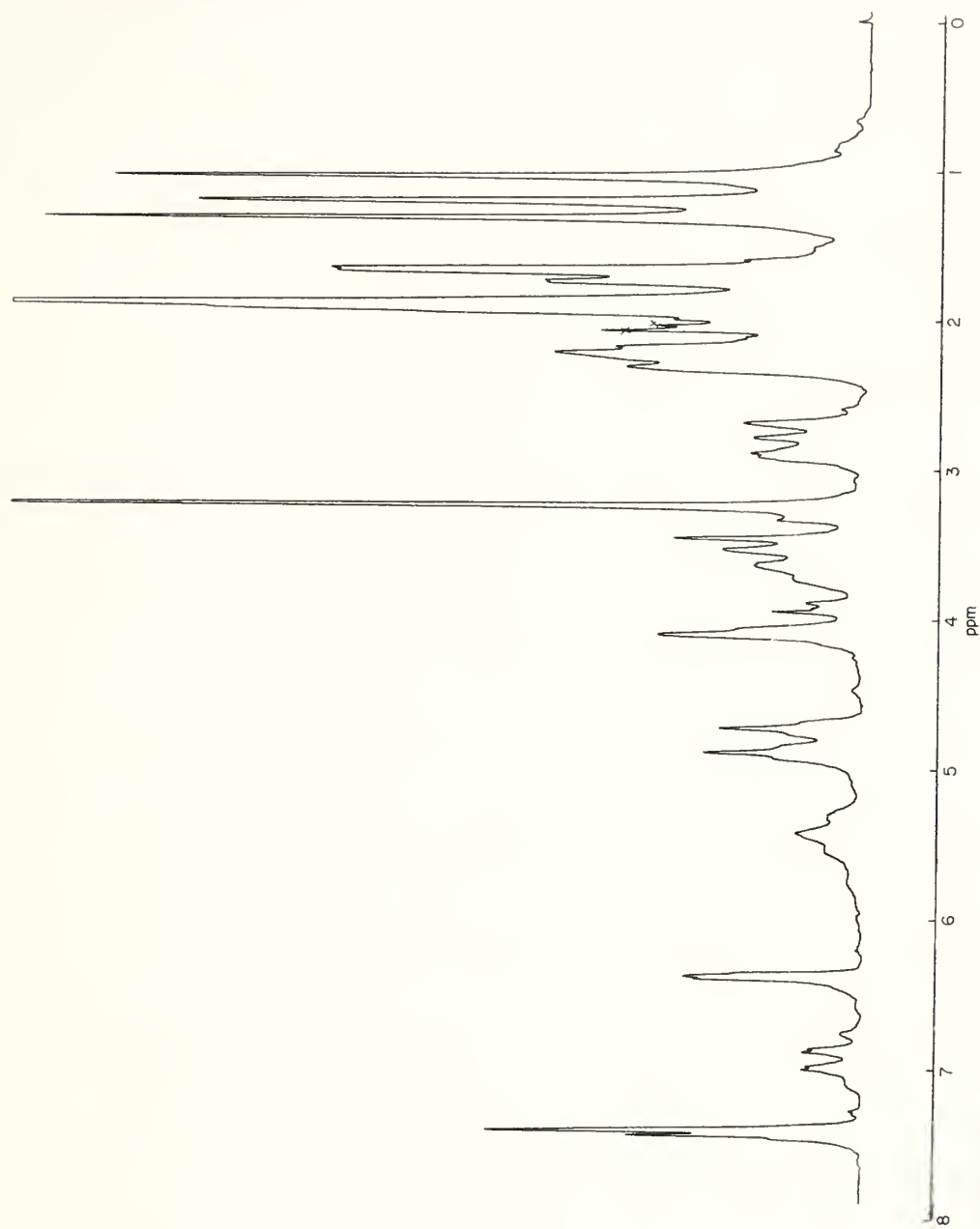


Figure 5.---60-MHz nmr spectrum (x = solvent) of house fly feeding deterrent.

Table 1.--Results of bioassay of candidate substances as house fly deterrents for test/control counts (test materials added to sucrose)

Candidate substance	Age of house flies	Concentration (percent)			
		1.0	0.5	0.25	0.1
	Days	Test/control count			
Salannin (I)	9	—	0/30	3/30	0/38
N,N-Diethyl-m-tolamide (deet) ^{1/}	5	—	0/28	—	15/28
ZBecon 0759 (15% MGK ^{1/} /326 ^{2/})	6	—	0/25	0/30	0/20
Zoecon 0760 (15% S. C. Johnson R-69 ^{3/})	6	—	0/20	0/25	0/33
Cedrene ^{4/}	4	0/38	2/38	—	—
Sandalwood oil ^{5/}	4	5/30	7/30	—	—
Gurjon balsam oil ^{5/}	4	9/19	3/17	—	—
Angelica seed oil ^{6/}	4	4/16	2/23	—	—
Terpenes of rectified sweet orange ^{7/}	4	18/18	8/15	—	—
Limonene ^{4/}	5	0/30	7/33	—	—
β-Caryophyllene ^{8/}	10	3/33	4/30	—	—
Amorphene	10	2/35	3/33	—	—
(Z)-9-Tricosene (muscalure)	5	25/23	30/23	—	—

^{1/} McLaughlin-Gormley-King.

^{2/} Dipropyl 2,5-pyridinedicarboxylate.

^{3/} 3-Acetyl-2-(2,6-dimethyl-5-heptenyl)oxazolidine.

^{4/} Aldrich Chemical Co.

^{5/} Dodge & Olcott Inc.

^{6/} Magnus Mabee & Reynard, Inc.

^{7/} Camilli, Albert et Laloue.

^{8/} K & K Fine Chemicals.

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